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THE MECHANISM OF RHODOPSIN SYNTHESIS

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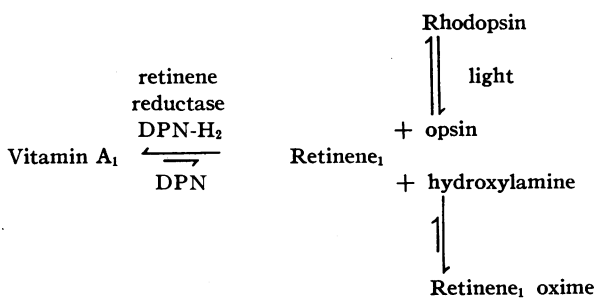
We have recently demonstrated the synthesis of rhodopsin from vitamin A_1 and opsin (rhodopsin-protein) in retinal homogenates and aqueous retinal extracts.[1] This process was shown to be aided by supplementation with homogenates of the pigment layers of the eye—the pigment epithelium and choroid—and with cozymase (DPN). A mechanism was proposed for rhodopsin synthesis, based upon the following considerations.

(1) Vitamin A_1 is in equilibrium with retinene$_1$ through the retinene reductase system, in which cozymase functions as coenzyme.[2] The equilibrium lies far over toward the side of reduction (vitamin A_1), but can be displaced in the oxidative direction by the use of a retinene-trapping reagent such as hydroxylamine, which condenses spontaneously with retinene$_1$ to form retinene$_1$ oxime. In this process the endergonic oxidation of vitamin A_1 to retinene$_1$ is coupled with the exergonic trapping reaction.[1]

(2) Retinene$_1$ condenses spontaneously also with opsin to form rhodopsin.[3] This exergonic process can serve as a retinene-trapping reaction in the retina. Rhodopsin may therefore be synthesized by the oxidation of vitamin A_1 to retinene$_1$ by the retinene reductase system, coupled with the condensation of retinene$_1$ with opsin to form rhodopsin.[1]

These reactions are summarized in the following diagram:



In this system the oxidation of vitamin A_1 to retinene$_1$ is the limiting process. It is presumably here that DPN acts in promoting rhodopsin synthesis.

In the experiments to be described we have examined systematically the effects on rhodopsin synthesis of factors which promote the oxidation of vitamin A_1. We have analyzed in part the contribution of the pigment epithelium. Finally, we have assembled a minimal system of well-defined components, which synthesizes rhodopsin from vitamin A_1 in aqueous solution.

Procedures.—The retinas used in these experiments were dissected from the eyes of dark adapted frogs (*Rana pipiens*) under dim red light. The pigment layers of the eye—the combined pigment epithelium and choroid layer—were collected separately. The retinas were bleached by exposure to an intense tungsten source, filtered through Corning glasses 3387 and 3966 to remove heat, and violet and near ultraviolet radiation which might otherwise destroy vitamin A_1 and retinene$_1$. The retinas were left for 1–$1^1/_2$ hours in the light at room temperature, to allow all reactions to be completed. They were then colorless, all the retinene$_1$ liberated in the bleaching of rhodopsin having been reduced to vitamin A_1.

Preparations for the synthesis of rhodopsin were incubated in the dark at room temperature, usually overnight. Rhodopsin was extracted with 2 per cent crystalline digitonin in water, the digitonin acting as solubilizer. Digitonin was used also to bring vitamin A_1 or retinene$_1$ into aqueous solution.

Digitonin extracts were examined for the presence of rhodopsin by measuring the absorption spectrum in darkness, bleaching in bright tungsten light filtered through yellow Corning glass 3384, and re-measuring the absorption spectrum. The difference in absorption before and after bleaching —the so-called difference spectrum—identifies rhodopsin and measures its quantity. All the figures in the present paper are such difference spectra.

Opsin was prepared by methods similar to those used to make the purest preparations of rhodopsin.[3] Cattle retinas were bleached to colorlessness, disintegrated mechanically, and the outer segments of the rods were isolated by centrifuging in 35 per cent sucrose solution. The rods remain in suspension, while the remaining retinal tissues are sedimented. The suspension in sugar solution is diluted, and the rods collected by centrifuging. They are tanned in 4 per cent alum, and washed repeatedly with water and neutral phosphate buffer. Sometimes they are lyophilized, and extracted with petroleum ether. The residue of these treatments is free from water-soluble and fat-soluble material, and most of its remaining proteins have been rendered insoluble by the alum treatment. From this residue opsin is extracted into clear, colorless solution with 2 per cent aqueous digitonin.

Oxidative Factors: "Succinoxidase."—We have proposed that cozymase stimulates rhodopsin production by acting in the retinene reductase system,

in which it oxidizes vitamin A_1 to retinene$_1$, and is itself reduced in the process. If this view is correct, an enzyme system which re-oxidizes cozymase should aid in rhodopsin synthesis.

The so-called "succinoxidase" of heart muscle is of this nature. It is a particulate complex that includes all the components needed to transfer hydrogen from cozymase to oxygen: cytochrome oxidase, the cytochromes, and riboflavine enzymes. In our experiments we have used the beef heart

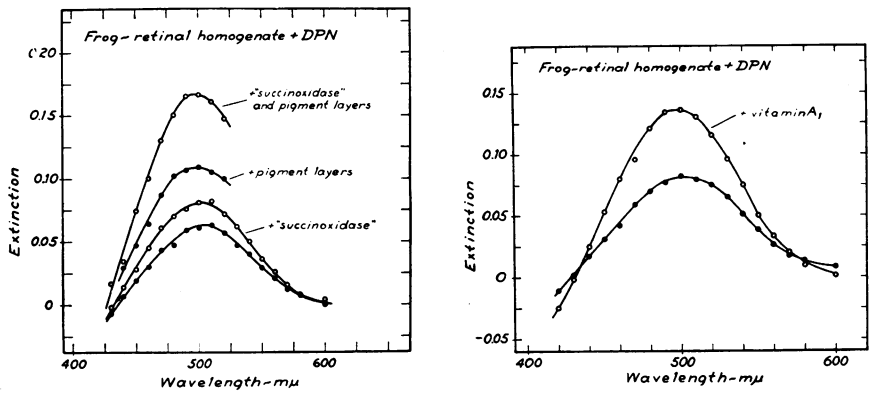


FIGURE 1

Stimulation of rhodopsin synthesis with "succinoxidase" and pigment layer homogenate. Frog retinal homogenate supplemented with DPN, and incubated alone, with "succinoxidase," with pigment layer homogenate, and with both "succinoxidase" and pigment layer homogenate. Difference spectra of rhodopsin extracted after incubation are shown. Each preparation contained 4.5 retinas, 1.5 mg. DPN, and 0.002 M NaF in a final volume of 1.0 ml. Each portion of pigment layer homogenate contained pigment layers of 9 eyes. "Succinoxidase" was supplemented with cytochrome *c*, 1.5×10^{-5} M.

FIGURE 2

Stimulation of rhodopsin synthesis by vitamin A_1. Frog retinal homogenate supplemented with DPN, and incubated alone and with added vitamin A_1. Difference spectra of rhodopsin extracted after incubation are shown.

preparation of Ball and Cooper,[4] supplemented with cytochrome *c* in a final concentration of 10^{-5} *M*.

Figure 1 shows the results of one such experiment. Retinas of 9 frogs were isolated and bleached to colorlessness (vitamin A_1 and opsin). The retinas were homogenized, supplemented with cozymase and divided into 4 equal portions. These were incubated in the dark at room temperature in the following way: one diluted only with buffer, the second with "succinoxidase," the third with a homogenate of the pigment layers from 9 eyes and the fourth with both "succinoxidase" and the same amount of pigment

layer homogenate. The final volume of each mixture was 1.0 ml. After incubation, all preparations were centrifuged, and the solid residues were extracted with 0.65 ml. of 2 per cent aqueous digitonin.

Figure 1 contains the difference spectra of these extracts. It shows that the addition of "succinoxidase" to the retinal homogenate increased the yield of rhodopsin 33 per cent, or 50 per cent in the presence of pigment layer homogenate. The latter roughly doubled the yield of rhodopsin, whether or not "succinoxidase" had also been added. These observations show that "succinoxidase" promotes the synthesis of rhodopsin from vitamin A_1, and that this effect is relatively independent of the action of the pigment layers.

In the mechanism of rhodopsin synthesis that we have proposed, such respiratory factors as "succinoxidase" participate only indirectly, by reoxidizing DPN-H_2. In the presence of excess DPN, such factors may not be required at all. Probably for this reason we have found in some experiments that removal of oxygen or poisoning with 10^{-3} M cyanide interferes relatively little with rhodopsin synthesis in retinal homogenates supplemented with DPN, or with the promotion of this synthesis by added pigment layer homogenate.

Addition of Vitamin A_1.—Figure 2 shows the results of a typical experiment. Right and left retinas of 5 dark adapted grass frogs were dissected into separate vessels and were bleached to colorlessness. One set of retinas was homogenized with 0.45 ml. neutral phosphate buffer and one drop of cottonseed oil containing 0.5 per cent of the antioxidant, α-tocopherol; the other set with an identical mixture in which the oil contained also 0.07 mg. vitamin A_1. To each homogenate, 1.8 mg. DPN was added, and both were incubated for 12 hours in the dark. They were centrifuged, and rhodopsin was extracted from the solid residues with 0.65 ml. of 2 per cent aqueous digitonin. The difference spectra of these extracts are shown in figure 2. The added vitamin A_1 had increased the synthesis of rhodopsin about 65 per cent.

We have found that a more effective way to add vitamin A_1 to such a system is to grind the oil containing the vitamin with a dry powder of lyophilized retinas. The powder is afterward suspended in buffer solution, and is incubated in the dark. Such an experiment is described below (Fig. 4).

Role of the Pigment Epithelium.—We have shown that the addition of pigment layer homogenate greatly increases the yield of rhodopsin in retinal homogenates, whether otherwise unsupplemented, or supplemented with DPN or "succinoxidase."

It might be supposed that the pigment layers themselves synthesize rhodopsin. We have, however, supplemented pigment layer homogenate strongly with vitamin A_1 and DPN, and could observe only a trace of rhodopsin formation, no more than might be explained by contamination

with a few rods. Pigment layers have also been tested for the presence of retinene reductase by incubating with DPN and hydroxylamine, the latter to act as a retinene-trapping reagent; no evidence was obtained of oxidation of vitamin A_1 to retinene$_1$. The pigment layers apparently do not form rhodopsin; they seem to lack opsin, and probably also retinene reductase.

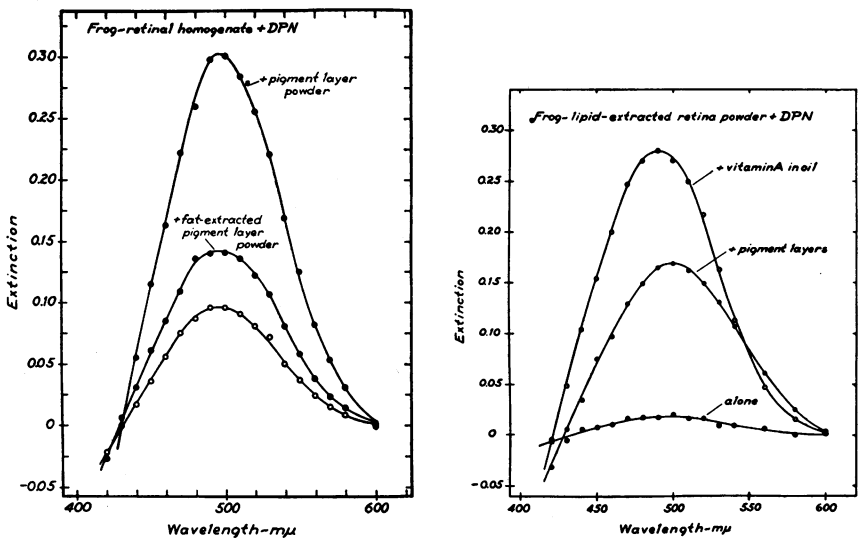


FIGURE 3

The fat-soluble factor of the pigment layers. Frog retinal homogenate supplemented with DPN was incubated alone, with a brei of lyophilized pigment layers, and with a similar brei of lyophilized pigment layers which had previously been extracted with petroleum ether. Difference spectra of rhodopsin extracted after incubation are shown. The fat-extracted pigment layers had lost most of their capacity to stimulate rhodopsin synthesis.

FIGURE 4

Synthesis of rhodopsin from extra-retinal vitamin A_1. Lyophilized retinas were extracted with petroleum ether to remove vitamin A_1, and were supplemented with DPN in neutral phosphate buffer. Equal portions were incubated alone, with lyophilized pigment layers, and with vitamin A_1 in oil. The fat-extracted retinas make almost no rhodopsin. The rhodopsin formed in the other preparations is derived therefore from the vitamin A_1 added in oil or from the pigment layers. About 5 times as much vitamin A_1 was added in oil as was contained in the pigment layers.

The effectiveness of the pigment layers in our experiments is due principally to a fat-soluble factor. If pigment layers are extracted with petroleum ether, they lose most of their capacity to stimulate rhodopsin synthesis. This is shown in figure 3.

A retinal homogenate which had been supplemented with DPN was incubated in the dark (*a*) alone; (*b*) with 6.3 mg. of a powder of lyophilized pigment layers; and (*c*) with 6.3 mg. of the same pigment layer powder which had been extracted twice with petroleum ether. After 12 hours incubation, rhodopsin was extracted from all three portions with digitonin solution. Figure 3 shows the difference spectra of these extracts. The addition of lyophilized pigment layers to the retinal tissue had tripled the yield of rhodopsin (*b*); but this effect was very much reduced on extraction with petroleum ether (*c*). The same result is obtained when the extraction is performed at −15°C.

The fat-soluble factor of the pigment layers is vitamin A_1. The pigment layers contain a large quantity of vitamin A_1, in this species about 4 μg. per eye, concentrated almost wholly in the pigment epithelium.[5] A simple experiment shows that in our homogenates, vitamin A_1 is transferred from the pigment epithelium to the retinal tissue for rhodopsin synthesis.

Dark adapted retinas of 8 frogs were bleached to colorlessness, lyophilized, ground to a powder, and extracted thoroughly with petroleum ether to remove vitamin A_1. The dry powder was divided into three equal portions, and treated as follows: portion (*a*) was left unsupplemented; (*b*) was stirred with 0.2 ml. cottonseed oil containing 0.27 mg. vitamin A_1 and 1 mg. α-tocopherol; and (*c*) was mixed with a lyophilized powder of 12 pigment layers. Each portion was stirred with 0.33 ml. phosphate buffer, pH 6.8, containing 1.2 mg. DPN, and was incubated for 12 hours in the dark. It was then extracted with 0.65 ml. of 2 per cent digitonin solution, and the difference spectra of these extracts were measured.

Figure 4 shows the results of this experiment. From the very small synthesis of rhodopsin in (*a*), it is clear that extraction of the retinal tissue with petroleum ether had removed almost all vitamin A_1. Therefore virtually all the rhodopsin in (*b*) and (*c*) must have been made from extra-retinal vitamin A_1. In (*b*) the vitamin A_1 was supplied as a solution in oil, and had resulted in a 40 per cent yield of rhodopsin. In (*c*) the vitamin A_1 for rhodopsin synthesis was supplied by the lyophilized pigment layers, and had resulted in a 25 per cent yield. It should be noted that the amount of vitamin A_1 added in oil in (*b*) was about 5 times as great as the pigment layers contained in (*c*).

This experiment shows unequivocally that in homogenates the vitamin A_1 of the pigment epithelium is available for rhodopsin synthesis by the retinal tissue. It shows also that supplementation of retinal tissue with vitamin A_1 can substitute for supplementation with whole pigment layers. It should be recalled that these experiments are all conducted in the presence of excess DPN. Under these circumstances it seems that the primary action of the pigment layers is to supply the retinal tissue with vitamin A_1.

This, however, is not their only contribution to rhodopsin synthesis. The activity of frog pigment layers is greatly reduced by heating for 2–3 minutes at 100°C., though they still contain large amounts of vitamin A_1. From homogenized pigment layers, deep yellow, opalescent solutions can be prepared in neutral M/15 phosphate buffer, which strongly stimulate the synthesis of rhodopsin in retinal homogenates, though they contain very little vitamin A_1. The activity of such solutions is destroyed by boiling for 2–3 minutes. They contain heat-labile factors, probably proteins, which aid in rhodopsin synthesis. Their yellow color is due principally to riboflavine. Their composition and action are being examined further.[6]

It must not be forgotten also that in the intact eye the layer of rods and cones depends primarily upon the choroidal circulation for its exchanges with the blood, and receives oxygen and metabolites from this source by diffusion through the intervening pigment epithelium. In cold-blooded animals, the retina has no blood vessels, and except for the sparse hyaloid vessels, depends entirely upon the choroidal circulation. In mammals, the retinal blood vessels supply primarily the inner retinal layers, and the sensory epithelium still depends principally upon the choroid.[7] These are added reasons why the normal functioning of the rods and the synthesis of rhodopsin *in vivo* demand close contact between the retina and the pigment epithelium.

Synthesis of Rhodopsin in a System of Known Components.—If our assumptions concerning the mechanism of rhodopsin synthesis are valid, it should be possible to perform this synthesis in a system containing only four components: vitamin A_1, retinene reductase, cozymase and opsin.

Of these substances, only retinene reductase is not yet available in highly purified condition. Bliss, however, has reported that the equilibrium between vitamin A_1 and retinene$_1$ is catalyzed by crude preparations of liver alcohol dehydrogenase, with DPN as coenzyme.[8] We have confirmed this observation, using the crystalline alcohol dehydrogenase of horse liver, prepared by Bonnichsen.[9] Dr. Warren Yudkin in our laboratory has found also that a homogenate of frog retinas, supplemented with cozymase, oxidizes ethyl alcohol. It is possible therefore that retinene reductase is identical with liver alcohol dehydrogenase. The relation between these enzymes is being examined further.[10]

We have assembled in aqueous digitonin solution the four components which should constitute a minimal system for the synthesis of rhodopsin: vitamin A_1, concentrated from fish liver oils; crystalline alcohol dehydrogenase from horse liver; cozymase (Sigma, 90 per cent pure); and purified opsin, prepared as described above. As control, an identical mixture was prepared which lacked only the alcohol dehydrogenase.

Figure 5 shows the results of such an experiment. In this instance the control mixture generated a small amount of rhodopsin, the source of which

is not yet clear. In the complete mixture, however, rhodopsin is synthesized with high efficiency. Indeed, the opsin used in this experiment, when mixed with excess retinene$_1$, yielded almost exactly as much rhodopsin as was formed from vitamin A$_1$. It seems therefore that the yield of rhodopsin in this experiment was limited only by the amount of available opsin.

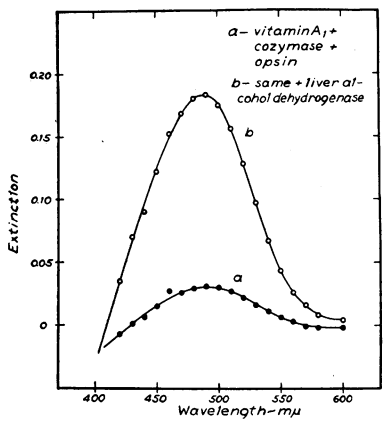


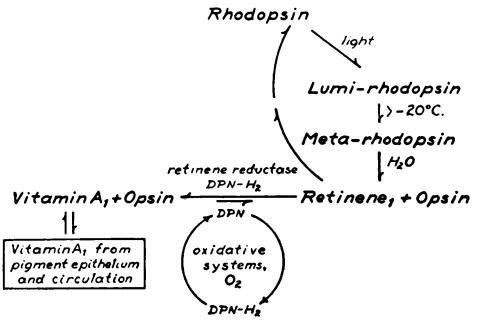
FIGURE 5

Synthesis of rhodopsin in a solution of known components. The upper curve shows the difference spectrum of rhodopsin synthesized by incubating together: vitamin A$_1$, 0.35 mg. in 0.08 ml. cottonseed oil containing 0.5 per cent α-tocopherol; crystalline horse liver alcohol dehydrogenase, 0.6 mg.; cozymase, 0.2 mg.; and frog opsin in 2 per cent digitonin. Final volume, 0.8 ml., in neutral phosphate buffer. The lower curve shows the rhodopsin formed in an identical mixture lacking only the alcohol dehydrogenase. The difference spectra were measured in the presence of hydroxylamine, 0.17 *M*, to block regeneration. The opsin used in this experiment, incubated separately with excess retinene$_1$, yielded rhodopsin with a maximal density of 0.18, almost exactly the same amount as was synthesized here from vitamin A$_1$.

Discussion.—The net result of our experiments is to confirm the view that rhodopsin is synthesized by the oxidation of vitamin A$_1$ to retinene$_1$, coupled with the condensation of retinene$_1$ with opsin. All factors known to promote the oxidation of vitamin A$_1$ aid in the synthesis of rhodopsin: vitamin A$_1$, cozymase and oxidative systems, such as "succinoxidase," which keep cozymase oxidized. The pigment epithelium has been shown to stimulate rhodopsin synthesis in our experiments primarily by supplying one of these factors, vitamin A$_1$, to the retinal tissue. The water-soluble, heat-labile factor of the pigment epithelium is still to be identified, and its function determined. The proposed mechanism, however, is established unequivocally with the demonstration that rhodopsin is synthesized in solution in the system of known components just described.

Is this the only pathway of rhodopsin synthesis? The only reply to this question possible at present is that the mechanism we have described may well be adequate, and that there is as yet no evidence of an alternative mechanism.

The known reactions that compose the rhodopsin system can therefore be formulated as follows:



The right-hand portion of the diagram indicates that all the intermediate stages in bleaching may not be retraced when retinene$_1$ and opsin recombine to form rhodopsin.

Of the four components from which the rhodopsin system has been assembled *in vitro*—vitamin A_1, retinene reductase or alcohol dehydrogenase, cozymase and opsin—only the last appears to be confined to the retina, and indeed to the outer segments of the rods. All the other components have a wider distribution in tissues, and were obtained in our experiments from extra-retinal sources. The specific factor that probably restricts the synthesis of rhodopsin to rods, and limits its quantity there, is opsin.

It is important to note that the rhodopsin system, though isolable *in vitro* as we have described, maintains basic connections *in vivo* with the circulation and the general metabolism. Some years ago it was shown that during light adaptation *in vivo*, the retina loses vitamin A_1 by diffusion into the pigment epithelium and the blood; and that it recaptures vitamin A_1 during dark adaptation by binding it in non-diffusible form in rhodopsin.[11] These interchanges, confirmed and reemphasized in the present experiments, connect the rhodopsin system with the metabolism of vitamin A_1 throughout the organism, and with a more remote circumstance, its nutritional supply.

Through its dependence upon cozymase, the rhodopsin system is connected also with the general processes of retinal respiration and fermentation; and these in turn depend upon the blood circulation for supplies of oxygen and metabolites. The retinal metabolism determines not only the oxidation-reduction state of cozymase, but probably must maintain its concentration by continuous synthesis, for cozymase appears to be under constant attack by cellular nucleotidases.[12] The rhodopsin system, therefore, though it can be isolated in solution with four essential components, makes multiple connections *in vivo* with the circulation and the general metabolism.

Summary.—It was proposed earlier that rhodopsin is synthesized by the oxidation of vitamin A_1 to retinene$_1$ by cozymase (DPN) and retinene

reductase, coupled with the condensation of retinene$_1$ with opsin (rhodopsin-protein) to form rhodopsin. The present experiments confirm this view. There is as yet no evidence for the formation of rhodopsin by another mechanism.

Factors which promote the oxidation of vitamin A_1 by retinal homogenates, all are shown to aid the synthesis of rhodopsin. These include cozymase, vitamin A_1 and oxidative systems such as heart particle "succinoxidase" which keep cozymase oxidized.

The pigment epithelium of the eye is shown to promote rhodopsin synthesis by supplying vitamin A_1 to the retinal tissue. The pigment layers of the eye contain also a heat-labile, water-soluble factor, still unidentified, that stimulates the synthesis of rhodopsin. Through cozymase, the rhodopsin system is connected with the general retinal metabolism, and this in turn depends for supplies of oxygen and metabolites upon exchanges with the blood circulation of the choroid, by diffusion through the intervening pigment epithelium.

A system has been assembled from four well-defined components, which synthesizes rhodopsin from vitamin A_1 in solution. It contains vitamin A_1, cozymase, opsin and crystalline liver alcohol dehydrogenase which substitutes for retinene reductase.

* This research was supported in part by a grant from the Medical Sciences Division of the Office of Naval Research. A preliminary account of most of these experiments was presented at the 18th International Physiological Congress in Copenhagen, August 15–18, 1950 (*Abstracts of Communications*, p. 264).

[1] Wald, G., and Hubbard, R., PROC. NATL. ACAD. SCI., **36**, 92 (1950).

[2] Wald, G., and Hubbard, R., *J. Gen. Physiol.*, **32**, 367 (1948–1949); Wald, G., *Science*, **109**, 482 (1949); *Biochim. et Biophys. Acta*, **4**, 215 (1950).

[3] Wald, G., and Brown, P. K., PROC. NATL. ACAD. SCI., **36**, 84 (1950).

[4] Ball, E. G., and Cooper, O., *J. Biol. Chem.*, **180**, 113 (1949). We are very grateful to these workers for a sample of their "succinoxidase" preparation.

[5] Wald, G., *J. Gen. Physiol.*, **19**, 351, 781 (1935–1936).

[6] Bliss has mentioned in a recent abstract that a heat-labile factor, which he says can be extracted from the pigment layers of the eye with 0.5 *M* NaCl, promotes the regeneration of rhodopsin after bleaching in digitonin solution (Bliss, A. F., *Fed. Proc.*, **9**, 12 (1950)). The process in question apparently does not involve vitamin A_1, and seems to be concerned with the regeneration of rhodopsin from retinene$_1$ and opsin. The connection with the present observations is problematical.

[7] Weiss, O., *Kurzes Handb. d. Ophth.*, **2**, 1 (1932).

[8] Bliss, A. F., *Biol. Bull.*, **97**, 221 (1949).

[9] Bonnichsen, R. K., *Acta Chem. Scand.*, **4**, 715 (1950). We should like to thank Dr. Bonnichsen for a gift of crystalline liver alcohol dehydrogenase, and for his assistance with preliminary experiments, while one of us (R. H.) was a guest at the Carlsberg Laboratory in Copenhagen last summer.

[10] It is noteworthy in this connection that yeast alcohol dehydrogenase, prepared by a modification of Racker's method (Racker, E., *J. Biol. Chem.*, **184**, 313 (1950)) does not appear to catalyze the retinene$_1$-vitamin A_1 equilibrium (R. K. Bonnichsen, personal communication). It is interesting also that monoiodoacetate (0.002 *M*), which is

known to inhibit the yeast enzyme, does not inhibit either liver alcohol dehydrogenase or retinene reductase.

[11] Wald, G., *J. Gen. Physiol.*, **19**, 781 (1935–1936); *Biol. Symp.*, **7**, 43 (1942).

[12] Mann, P. J. G., and Quastel, J. H., *Biochem. J.*, **35**, 502 (1941); Handler, P., and Klein, J. R., *J. Biol. Chem.*, **143**, 49 (1942); McIlwain, H., and Rodnight, R., *Biochem. J.*, **44**, 470 (1949).

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*POLYPLOIDY IN YEAST AND ITS BEARING ON THE OCCURRENCE OF IRREGULAR GENETIC RATIOS**

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The significance of irregular ratios, i.e., phenotypic ratios of 4:0, 3:1, 1:3, and 0:4 in asci obtained from crosses which are expected to give 2:2 segregations and commonly do so, has been the subject of considerable speculation in yeast genetics. Lindegren[1] has cited such irregularities as evidence that a gene can be affected by its allele when the two are present in the same cell. On this interpretation, the 4:0 and 3:1 asci are those in which the dominant gene has converted the recessive allele to the dominant condition; the 1:3 and 0:4 cases are indicative of the degradation of the dominant by the recessive allele.

The gene-conversion hypothesis does not lend itself at the present time to critical test, and therefore depends for its validation on the exclusion of other explanations based on known genetic mechanisms. To this end Lindegren and his colleagues have shown that neither multiple factors nor orthodox mutation can provide a general explanation for irregular ratios. Mundkur[2] has demonstrated the inadequacy of the Winge and Roberts[3] hypothesis that accounts for these ratios in terms of an extra mitosis in the formation of ascospores. Finally, Mundkur[4] has excluded polyploidy as a factor in this problem on the grounds that it will not account for all of the irregularities that have been found.

The evidence presented below that polyploidy does in fact occur in yeast has prompted us to reappraise its possible role in the interpretation of irregular ratios.

Evidence of Diploid Ascospores.—Among 64 four-spored linear asci whose spores were tested for mating type, galactose fermentation, and growth habit in liquid medium, one ascus was found which exhibited an irregular ratio for each of these characters. The asci were obtained from a cross between two clones of *Saccharomyces* that were presumably haploid and of composition *agf* and *αGF*, respectively.[5] Clones were grown from the four